Smoothelin Expression in the Gastrointestinal Tract: Implication in Colonic Inertia

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Abstract: Colonic inertia is a frustrating motility disorder to patients, clinicians, and pathologists. The pathogenesis is largely unknown. The aims of this study were to: (1) characterize the expression of smoothelin, a novel smooth muscle-specific contractile protein expressed only by terminally differentiated smooth muscle cells, in the normal gastrointestinal (GI) tract; and (2) determine whether smoothelin is aberrantly expressed in patients with colonic inertia. A total of 57 resections of the normal GI tract (distal esophagus to left colon) were obtained from patients without GI motor dysfunction. Sixty-one colon resections were obtained from patients with a clinical diagnosis of colonic inertia. Smoothelin immunostaining was conducted on full-thickness tissue sections. In the nondysmotile controls, strong and diffuse cytoplasmic staining for smoothelin was observed in both the inner circular and outer longitudinal layers of the muscularis propria (MP) throughout the entire GI tract. The muscularis mucosae (MM) and muscular vessel walls were either completely negative or only patchily and weakly stained. The 1 exception to this pattern was observed in the distal esophagus, in which the MM was also diffusely and strongly stained. In cases with colonic inertia, a moderate to marked reduction of smoothelin immunoreactivity was observed in 15 of 61 (24.6%) colon resections, selectively seen in the outer layer of the MP. The data demonstrate that smoothelin is differentially expressed in the MP and MM of the normal GI tract and suggest that defective smoothelin expression may play a role in the pathogenesis of colonic inertia in a subset of patients.

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Chronic constipation is a frequent complaint, being reported in up to 16% of women and 12% of men.[1] Clinically, constipation and colonic motility disorders are defined by not only infrequent bowel movements, but also other manifestations such as excessive straining, hard stools, incomplete evacuation, sensation of obstruction, and manual maneuvers to facilitate defecation.[2,3] General management of constipation includes conservative therapies, such as behavioral modification, bulking agents, osmotic laxatives, wetting agents, stimulant laxatives, and biofeedback therapy. Even colonic pacing has been suggested as a therapeutic option.[4] Some medically refractory cases require surgical treatment with partial or total colectomy. The best surgical results occur when the motor disturbance is confined to the colon without generalized neuromuscular dysfunction of the gut.[5–9]

A variety of conditions can lead to colonic inertia or intestinal motility disorders, including structural, mechanical, metabolic, or functional causes[10]; but the underlying pathophysiology remains largely unknown. Recent studies have emphasized the role of interstitial cells of Cajal (ICC), the pacemaker of the gastrointestinal (GI) tract.[11] Using c-kit (CD117) immunohistochemical staining, numerous studies have shown a complete absence or significant reduction in the number of ICC in colon specimens resected from patients with colonic inertia when compared with normal controls.[12–20] However, discordant findings have also been reported. For example, the study by Toman et al[21] demonstrated no relationship between ICC numbers and chronic constipation when compared with controls. Furthermore, a significant reduction in the number of ICC was observed in patients with megacolon but without constipation.[15] It is interesting that several investigators have also examined the myenteric and submucosal plexuses using immunohistochemical staining for a neuronal marker, protein gene product 9.5 (PGP9.5), and reported neuropathologic alterations characterized by reduced ganglionic density and size (hypoganglionosis)

in patients with colonic inertia, when compared with normal controls.[12,13,15,19,20]

Smoothelin is a novel cytoskeletal protein that is selectively expressed in terminally differentiated, contractile smooth muscle cells.[22,23] The protein is encoded by the *SMTN* gene located on human chromosome 22q12[24] and has 2 isoforms generated by 2 physically separated promoters. The 59 kDa smoothelin-A is found in the smooth muscle of visceral tissues, such as the digestive tract, urinary bladder, and prostate, whereas the 110 kDa smoothelin-B is present in vascular smooth muscle.[25,26] The function of smoothelin is not entirely understood, but it interacts with filamentous α-smooth muscle actin and may thus serve a role in the contractile apparatus of smooth muscle cells.[27,28] In a mouse model, smoothelin-A is essential to intestinal contractile function. In comparison with wild-type littermates, smoothelin-A knockout mice exhibited fragile and less flexible intestines, impaired intestinal contraction, and hampered intestinal transit, which led to bowel obstruction, diverticulosis, and premature death, likely as a result of starvation and/or bowel perforation.[29] These observations are similar to those seen in patients with chronic intestinal pseudo-obstruction. In contrast, smoothelin-B knockout mice showed reduced arterial contractility, which led to hypertension and cardiac hypertrophy, suggesting a key role in normal cardiovascular function.[30]

Given its importance in smooth muscle contractility, we were interested in further investigating the potential role of smoothelin in the pathogenesis of intestinal dysmotility. In the current study, we examined smoothelin expression in the normal GI tract and in patients with a clinical diagnosis of colonic inertia.

MATERIALS AND METHODS

Specimens

This retrospective study involved GI specimens from patients who underwent surgical resection at the authors' institutions. For the normal group, a total of 57 surgically resected specimens were included (distal esophagus 3, stomach 4, duodenum 12, jejunum 7, ileum 11, right colon 10, and left colon 10). These were typically margin sections selected from specimens resected for tumors. None of the patients had a clinical history of GI motility disorders. The proximal esophagus was excluded from the study because the muscularis propria (MP) in that location consists of striated muscle rather than smooth muscle. For the dysmotility group, a total of 61 colon resections were selected based on clinical history. All the patients carried a clinical diagnosis of chronic constipation, slow transit constipation, colonic inertia, colonic motility disorder, or chronic intestinal pseudo-obstruction. The disorders in all the patients were thought to be idiopathic because extensive clinical workup had been performed, and all the potential etiologies had been ruled out. They underwent surgical resections due to failed responses to medical management. Of this group of patients, 22 colon resections contained an attached portion of the ileum, which was also included in the study. The study was approved by the Institutional Review Boards at Cedars-Sinai Medical Center and the coauthors' institutions.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue blocks that represented full-thickness and well-oriented sections were selected. Immunohistochemical detection of smoothelin was performed on 4-μm tissue sections using the Ventana Benchmark ULTRA automated stainer (Ventana Medical Systems, Tucson, AZ) following a previously described protocol with slight modifications.[31] Briefly, deparaffinized slides were pretreated with the Ventana on board antigen retrieval system, followed by incubation with a mouse monoclonal antibody (clone R4A) that reacts with both smoothelin-A and smoothelin-B (Abcam Inc., Cambridge, MA) at room temperature for 1 hour at a 1:1000 dilution. After incubation with a secondary antibody, immunoreactivity was visualized using the ultraView Universal DAB Detection Kit (Ventana). Hematoxylin was used for counterstaining.

Additional immunostaining was performed using a prediluted monoclonal antibody against α-smooth muscle actin (clone ASM-1) obtained from Leica Biosystems (Richmond, IL). The staining procedures were conducted using the Leica Bond-III automated stainer and the Novocastra Bond Polymer Refine Detection System (Leica). Antigen retrieval was not needed for this antibody.

For each batch of staining, there were a positive control comprised of a section of normal colon and a negative control in which the primary antibody was replaced by non–human-reactive mouse IgG. Cytoplasmic staining was considered positive. Staining intensity was evaluated as weak or strong for positively stained cases. The location of immunoreactivity, that is, muscularis mucosae (MM), inner and outer layers of the MP, and blood vessels, was also recorded.

RESULTS

Smoothelin Expression in the Nondysmotile GI Tract

Strong and diffuse cytoplasmic staining for smoothelin was observed in both the inner circular and outer longitudinal layers of the MP throughout the entire GI tract (Figs. 1A–D). There was no appreciable difference in the intensity of smoothelin immunoreactivity in the MP from all portions of the GI tract. In contrast, the MM of the stomach, duodenum, jejunum, ileum, right colon, and left colon showed either no smoothelin immunoreactivity or only patchy and weak staining (Figs. 1B–D). The MM of the distal esophagus was an exception, exhibiting strong and diffuse immunoreactivity for smoothelin, which was identical to that seen in the MP (Fig. 1A). Similar to the MM of the stomach and intestines, the muscular blood vessels (including those in the esophageal sections) showed negative or very weak smoothelin immunoreactivity. These findings are summarized in Table 1.

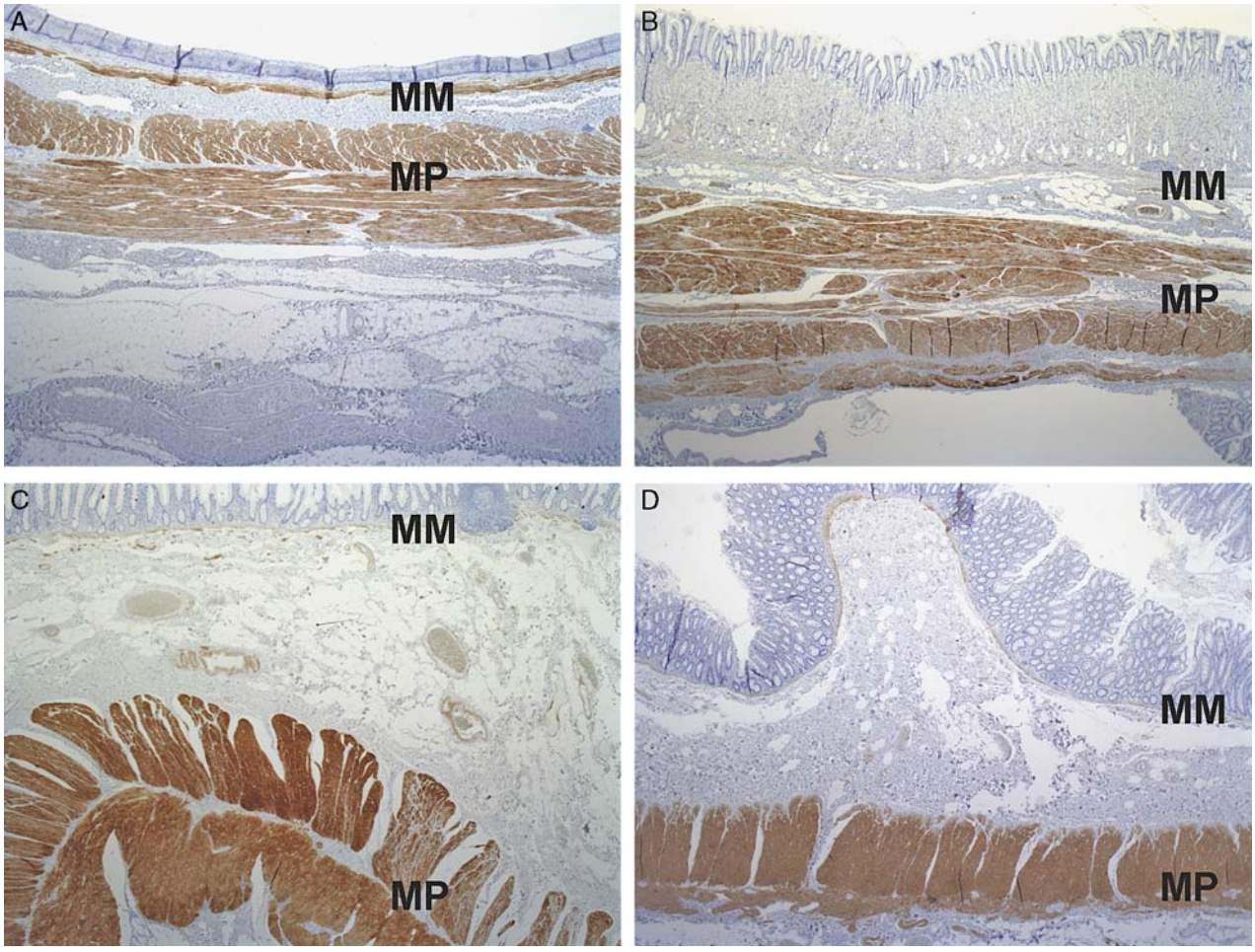

FIGURE 1. Smoothelin immunohistochemistry of the nondysmotile gastrointestinal tract. Full-thickness sections of the distal esophagus (A), stomach (B), small intestine (C), and large intestine (D) showed strong and diffuse staining in both inner circular and outer longitudinal layers of the muscularis propria (MP). The muscularis mucosae (MM) of the esophagus also showed strong and diffuse immunoreactivity. However, the MM of the stomach, small intestine, and large intestine were either negatively or only weakly stained (original magnification, ×20). full color online

Clinicopathologic Findings in Patients With Colonic Inertia

The patients with clinical diagnoses of colonic inertia ranged in age from 4 to 85 years (mean, 40 y; median, 39 y). There were 15 males and 46 females with a male to female ratio of 1:3.1. None of the patients had a documented history of GI neoplasm, malignancy in other parts of the body, inflammatory bowel disease, systemic neuromuscular diseases, systemic autoimmune disorders, or parasitic infestation involving the GI tract. Rectal prolapse, megacolon, and bowel perforation were reported in 4, 2, and 2 patients, respectively.

Surgical specimens consisted of 19 total colectomies, 18 subtotal colectomies, and 24 segmental colon resections. Of these, 22 specimens contained a variable length of attached ileum. On histologic examination, melanosis coli was noted in 11 cases, foci of cryptitis and occasional crypt abscesses in 3 cases, and pneumatosis coli in 1 case with perforation. Otherwise, no abnormal histologic findings were observed in the resected colons and ilea. Specifically, no degenerative changes or inflammatory cell infiltrates were appreciated in the submucosal and myenteric plexuses. Ganglion cells were present in all cases and did not appear reduced in number when compared with intestinal specimens resected from patients without intestinal motor dysfunction. The muscle fibers showed no evidence of degeneration, necrosis, atrophy, hypertrophy, inflammatory cell infiltration, or fibrous replacement (Figs. 2A, C).

Expression of Smoothelin and α-Smooth Muscle Actin in Patients With Colonic Inertia

Strong, diffuse, and uniform immunoreactivity for α-smooth muscle actin was observed in both inner and outer layers of the MP, MM, and submucosal muscular blood vessels in all 61 colon specimens resected from patients with colonic inertia (Figs. 2B, D). In contrast, a moderate to marked reduction in smoothelin immunoreactivity was detected in the outer longitudinal

TABLE 1. Summary of Smoothelin Immunostaining in the Nondysmotile Gastrointestinal Tract

	No. Specimens		
Site/Staining Intensity	**MM**	**MP**	**MBV**
Distal esophagus (n = 3)			
None/weak	0	0	3
Strong	3	3	0
Stomach (n = 4)			
None/weak	4	0	4
Strong	0	4	0
Duodenum (n = 12)			
None/weak	12	0	12
Strong	0	12	0
Jejunum (n = 7)			
None/weak	7	0	7
Strong	0	7	0
Ileum (n = 11)			
None/weak	11	0	11
Strong	0	11	0
Right colon (n = 10)			
None/weak	10	0	10
Strong	0	10	0
Left colon (n = 10)			
None/weak	10	0	10
Strong	0	10	0

MBV indicates muscular blood vessels; MM, muscularis mucosae; MP, muscularis propria.

layer of the MP in 15 of 61 (24.6%) colon resections for colonic dysmotility (Figs. 3B, D). The inner circular layer of the MP retained strong and diffuse smoothelin immunoreactivity, like that seen in control subjects with no colonic motor dysfunction. However, 2 of the patients with colonic inertia also had a patchy pattern of smoothelin labeling of the inner circular muscle layer (Fig. 3D). Nevertheless, the outer layer of the MP had reduced smoothelin immunoreactivity when compared with the inner layer. In comparison with nondysmotile control specimens, these findings were statistically significant (Table 2).

Of the 15 cases showing reduced smoothelin immunoreactivity in the outer layer of the colon, 7 had an attached portion of the ileum. A similar reduction in smoothelin immunoreactivity in the outer layer of the MP was observed in 3 of these 7 ileal resections (42.9%). The reduction was focal or patchy in all 3 cases. No abnormal smoothelin staining pattern was observed in the other 4 ileal specimens. In the remaining 15 ileal resections, which included the 2 cases showing only patchy weak smoothelin staining in the colon in both inner and outer layers of the MP, a normal smoothelin staining pattern in both layers of the MP was observed. Immunostaining for α-smooth muscle actin showed a moderate to marked reduction in staining intensity in 10 of 22 ileal resections (45.5%), exclusively seen in the inner layer of the MP. Of these 10 cases, 2 showed concurrently reduced smoothelin immunoreactivity in the colon but not in the ileum, and 3 demonstrated a reduction in both the colon and ileum. The remaining 5 cases showed a normal smoothelin staining pattern in both colon and ileal specimens.

DISCUSSION

In this study, we demonstrate that smoothelin is strongly, diffusely, and continuously expressed in both the inner circular and outer longitudinal layers of the MP throughout the entire GI tract. With the exception of the esophagus, weak and discontinuous smoothelin immunoreactivity is observed in the MM. In the esophagus, the MM is also strongly and diffusely stained. The muscular blood vessels are only weakly stained throughout the entire GI tract. These findings corroborate the observations reported by Coco et al[32] and Montani et al[33] and are consistent with the functional role of smoothelin in GI motility.[29] In addition, the differential staining patterns of smoothelin in the MP and MM are similar to those observed in the urinary bladder,[31,34–36] suggesting that smoothelin may serve as a useful diagnostic tool to help accurately assess the depth of tumor invasion in difficult cases.

There have been only 2 studies that have examined smoothelin expression in human specimens resected from patients with intestinal motility disorders. The study by Wedel et al[37] showed a lack of smoothelin immunoreactivity in 8 of 13 (61.5%) colon resections or full-thickness biopsies from patients with colonic inertia, 6 of 8 (75%) with idiopathic megacolon, and 6 of 10 (60%) with Hirschsprung disease. Interestingly, the lack of immunoreactivity was seen in both circular and longitudinal layers of the MP in all the cases, except for 1 case of colonic inertia, in which smoothelin immunoreactivity was lacking from the circular layer only. The lack of immunostaining was complete in half of the cases but focal or patchy in the remaining half. In the study by Amiot et al,[38] no abnormal smoothelin immunostaining pattern was observed in 35 intestinal samples (jejunum 13, ileum 17, colon 5) resected from 21 patients with idiopathic chronic intestinal pseudo-obstruction.

In the current study, we analyzed 83 intestinal samples (colon 61, ileum 22) from 61 patients with clinical diagnoses of colonic inertia and found reduced smoothelin immunoreactivity in one quarter of the colon specimens. The prevalence (24.6%) in our study is much lower compared with 61.5% reported by Wedel and colleagues ($P = 0.0178$ using a 2-tailed Fisher exact test). As Wedel et al[37] observed a complete or focal/patchy loss of smoothelin immunoreactivity in both layers of the MP, our study also demonstrated reduced staining intensity in the outer layer and patchy labeling of the inner layer in a subset of the patients who demonstrated decreased smoothelin reactivity. Although in 2 cases a much weaker staining intensity was observed in both layers in comparison with nondysmotile controls, none of our dysmotile cases showed selective loss of staining in the inner layer while retaining the normal staining pattern in the outer layer. We are not completely certain whether the markedly reduced smoothelin staining in both layers in these 2 cases is true or artifactual because no abnormal staining was observed for α-smooth muscle actin. Interestingly, the ileal resections in both cases exhibited a normal smoothelin staining pattern. Another different

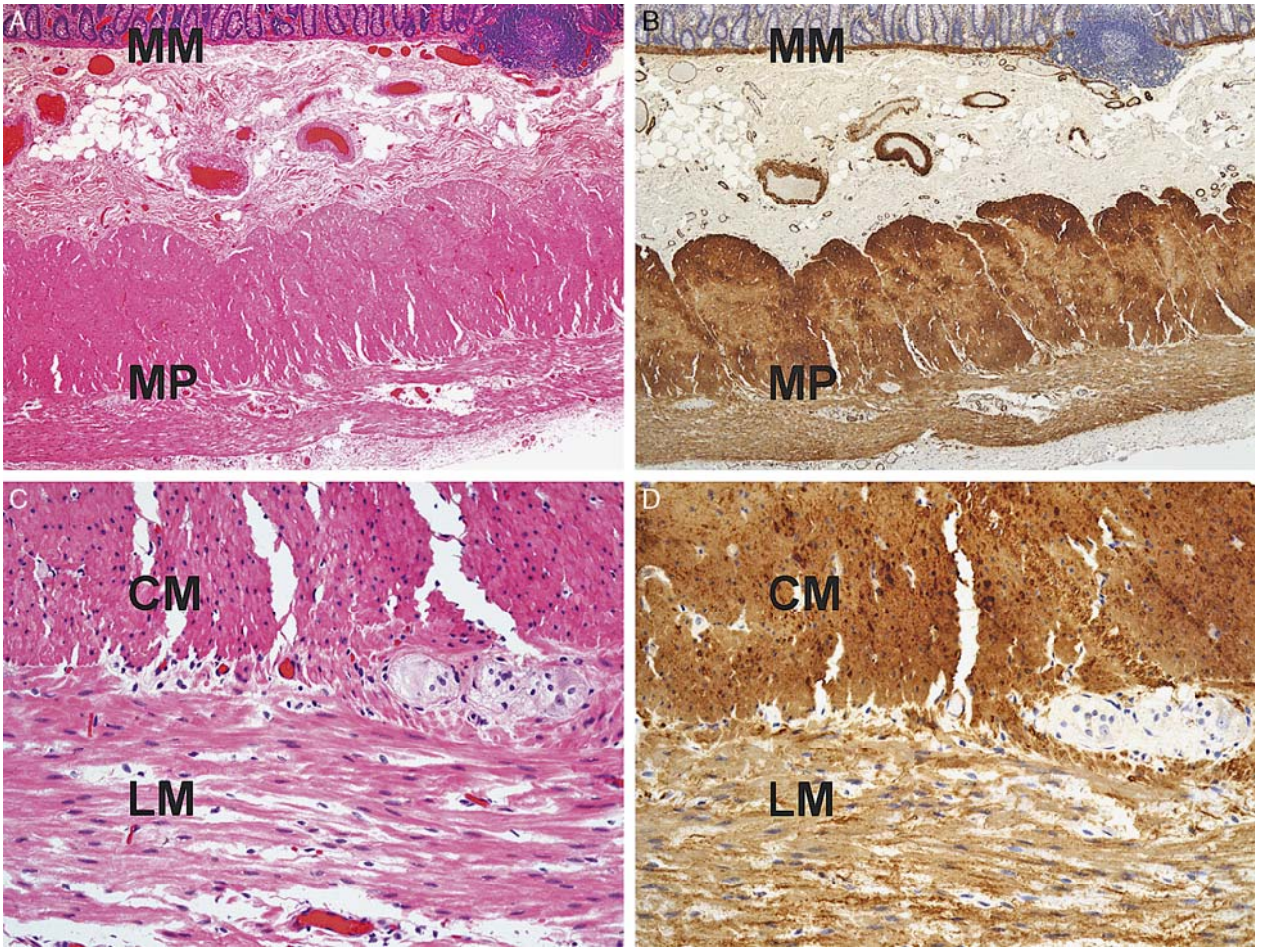

FIGURE 2. Histology and α-smooth muscle actin immunostaining in colon specimens resected from patients with colonic inertia. Hematoxylin and eosin staining showed intact muscularis mucosae (MM) and muscularis propria (MP) on the low-power view (A; original magnification, ×40) and unremarkable circular (CM) and longitudinal (LM) layers of the MP, as well as unremarkable myenteric plexus, on the high-power view (C; original magnification, ×200). Strong and diffuse immunoreactivity for α-smooth muscle actin was observed in the MM, MP, and submucosal blood vessels (B; original magnification, ×40). Both circular and longitudinal layers of the MP were equally stained (D; original magnification, ×200). full color online

observation reported by Wedel et al[37] is that the MM and blood vessels in their control colon specimens were also uniformly stained, with the staining intensity comparable with that of the MP. These findings are different than ours and those reported by others,[32,33] although the same monoclonal antibody (R4A) was used. The reason for these discrepancies is not readily apparent but may be related to different methods of specimen preparation. In the study by Wedel and colleagues, tissue samples were fixed in 4% paraformaldehyde solution at 4°C, cryopreserved in graded solutions of sucrose overnight, embedded in Tissue-Tek OCT, snap-frozen, and stored at −80°C. Sections for immunohistochemistry were cut on a cryostat at 15-μm thick and stored at −20°C until use. In contrast, our specimens were formalin-fixed and paraffin-embedded for routine histologic examination in several institutions. The patient populations might also be somewhat different. In those 13 colon specimens from patients with colonic inertia examined by Wedel et al,[37] hypoganglionosis and ICC deficiency were also present. In our cases, the density and morphology of ganglion cells appear normal by histologic examination in comparison with normal controls. In at least one third of the colon specimens included in this study, immunostaining for neuron-specific enolase was performed, which confirmed no reduction in the number of ganglion cells (data not shown). Immunostaining for c-kit was performed on all colonic and ileal specimens in our study, which demonstrated the presence of ICC in every case with a normal distribution pattern. By microscopic impression, the number of ICC did not appear to be reduced in dysmotile specimens when compared with normal controls (data not shown). A tedious quantitative analysis was not conducted for this study, however, due to technical difficulty of the analysis and because the focus of the study was smoothelin.

Nonetheless, the data presented in this study suggest that defective smoothelin expression may play a role in the pathogenesis of colonic inertia in a subset of patients.

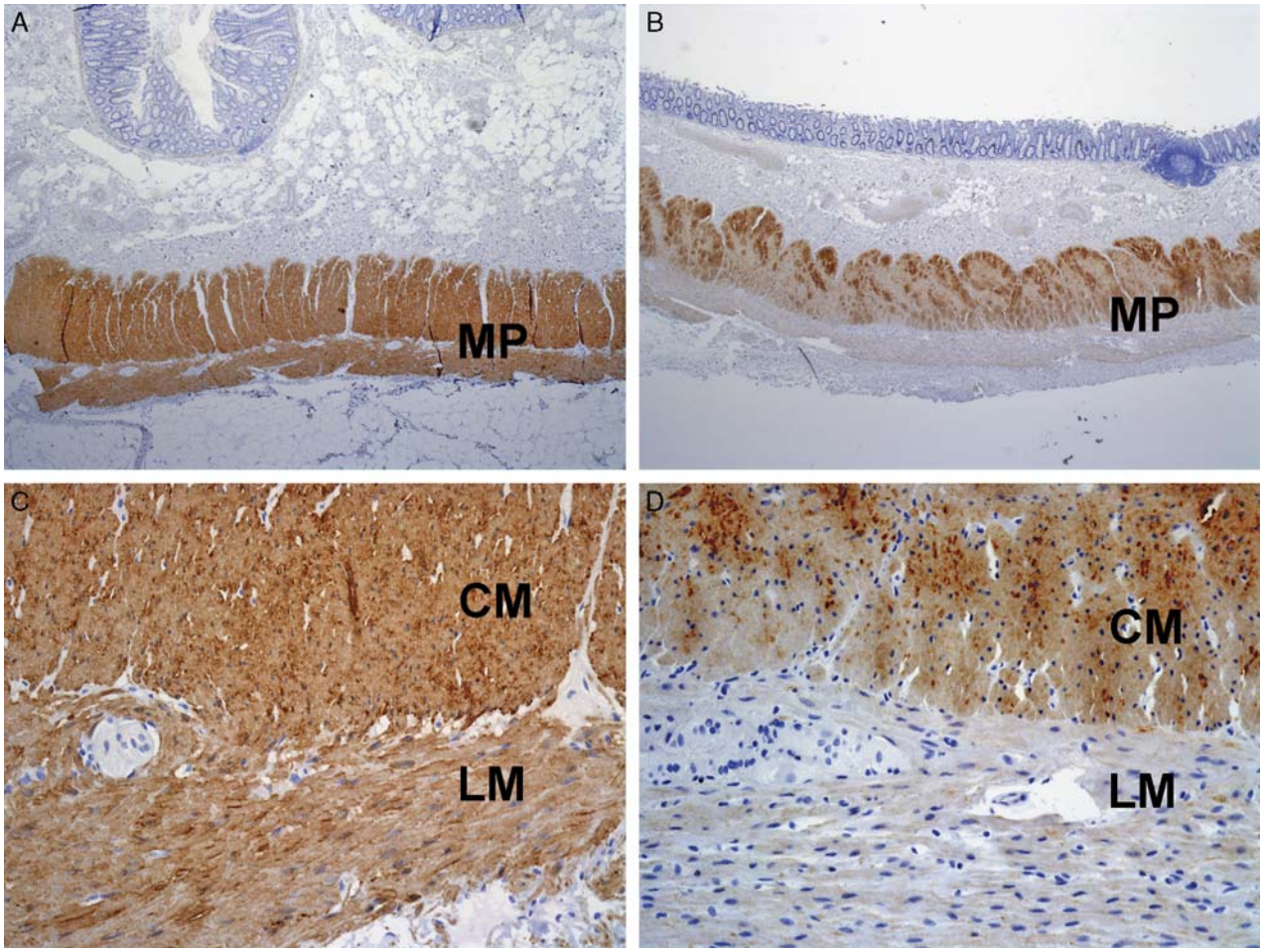

FIGURE 3. Smoothelin immunostaining in colon specimens resected from patients without (A, C) and with (B, D) intestinal motility disorders. Strong and diffuse immunoreactivity was observed in the muscularis propria (MP) of nondysmotile colons (A; original magnification, ×20), with both circular (CM) and longitudinal (LM) layers being equally stained (C; original magnification, ×200). In contrast, reduced smoothelin immunoreactivity was observed in a fraction of dysmotile colon specimens, selectively seen in the outer longitudinal layer of the MP (B; original magnification, ×20). A strong and diffuse staining pattern was well preserved in the inner circular layer. However, a subset of the patients with dysmotile colons demonstrated a patchy inner circular muscle layer (D; original magnification, ×200).

An interesting question one may ask is why the outer longitudinal layer of the MP is selectively affected. The answer to this question is unclear at the present but is likely to be related to the special functions of the longitudinal muscle layer in peristalsis of the intestine.[39,40] It is interesting to note that familial visceral myopathy (intestinal pseudo-obstruction) is histopathologically characterized by loss of the longitudinal muscle of the MP.[41]

Our study showed reduced smoothelin immunoreactivity in 3 of 22 ileum specimens from patients with colonic inertia. No abnormal smoothelin staining was observed in any small bowel specimens in controls with no motor dysfunction, although the difference is not statistically significant due to the small numbers of cases in both groups. The significance of this finding remains to be investigated in the small intestine. It is interesting to note, however, that a few studies have reported motor and neuropathologic abnormalities in the small bowel in patients with colonic inertia,[42–44] suggesting a generalized intestinal disorder in some patients. This may explain why some of the patients remain with impaired motor function even after total or subtotal colectomy.[9,45] Identification

TABLE 2. Comparison of Abnormal Smoothelin Immunostaining Between Specimens From Subjects With and Without Intestinal Motor Dysfunction

	No. Abnormal/Total Specimens*		
Specimens	**Dysmotility**	**Normal**	***P*****
Colon	15/61	0/20	0.0167
Ileum	3/22	0/11	0.5343

*Abnormal smoothelin expression was defined as reduced staining intensity in the outer layer of the muscularis propria. The total number of colon specimens for the normal group included both the right and left colons.

**A *P*-value was calculated by using a 2-tailed Fisher exact test. A *P*-value of <0.05 was considered statistically significant.

of histologic or immunohistochemical abnormalities, such as defective smoothelin expression, at the proximal margin sections may help assess postoperative prognosis and guide clinical management of the patients.

The abnormal staining pattern for α-smooth muscle actin observed in a subset of ileal specimens from patients with colonic inertia in our study is similar to that reported by others.[38,46,47] It remains debatable, however, whether this abnormal staining pattern represents a true causative effect or a nonspecific or even normal finding because the same staining pattern is also detected in a large proportion of nondysmotile small bowel specimens.[38,48] In our study, the absence of an abnormal staining pattern for α-smooth muscle actin in colon specimens argues against a causative role in the pathogenesis of colonic inertia.

In summary, the current work demonstrates reduced smoothelin immunoreactivity in the longitudinal layer of the MP of the colon in a subset of patients with colonic inertia. Given the important role of smoothelin in smooth muscle contractility, our findings suggest that defective smoothelin expression may contribute to the pathogenesis of some intestinal motility disorders.

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